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13. ABSTRACT (Maximum 200 Words)

Brain-metastasizing breast cancer is a major clinical problem. Cell-mediated immunotherapy is well-suited to attack it, but an appropriate small animal model was needed. In this final period (no-cost extension), we have worked to simplify and validate the model we have been developing. We present a novel model for visualizable, sporadic blood-borne micro-metastases of mammary carcinoma in the rat brain. We present evidence that tumor can enter the brain by vessels at all three relevant sites: vessels in the brain proper, in the meninges, and in the choroid plexus. In parallel, we describe how, during the course of this project, our own work and others' has changed our thinking about the best form of immunotherapy for blood-borne micro-metastases in the brain. We report that the work in the project has enabled us to obtain one new NIH grant, and is vital to a second one that is now being submitted. In a broader context, the PI's efforts to encourage fresh thinking about brain tumor trials ("Strengthening the Bridge from Bench to Bedside") are described.

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INTRODUCTION. This "idea grant" fulfilled its purpose in two ways. We did pursue our original ideas about brain-metastasizing breast cancer. In parallel, we gained insights that have led us to new ideas, not being tested by others.

Data from this project helped the PI to win a new R21 award from NIH, and is important for a second one that is being submitted (February 1). The project was also stimulating conceptually. During the course of the project, the PI edited two special issues for the *Journal of Neuro-Oncology*, one on brain tumor models, and one on brain tumor immunotherapy. Most recently, the PI proposed and then chaired a special 2-hour session on "Strengthening the Bridge from Bench to Bedside" at the November, 2004, Annual Meeting of the Society for Neuro-Oncology (SNO). (Citations are included in **Reportable Outcomes**, below.)

The work in this final report was done under a *no-cost extension*. The final report was delayed as we completed our validation of the model; we wanted to be sure that we had a viable model to offer, and had interpreted the tumor pattern correctly. We have now simplified the procedures, for both surgery and data handling, and confirmed that a new team of trainees could learn and perform them. We re-analyzed the existing data and added new rats. An abstract describing the model will be submitted for the coming Era of Hope meeting (2005).

Another delaying factor was a final illness and death in the PI's immediate family. The PI is grateful for the understanding of those with whom she has had personal contact.

BODY OF REPORT.

Overview. Our broad goals were: **1)** To make an appropriate rat model for blood-borne metastatic breast cancer in the brain (*tasks 1-3*). **2)** To use the model to test ideas about immunotherapy (*tasks 4-6*).

Overall, comments made by the original reviewers proved correct: That it would prove more difficult to develop the model than we were anticipating. In fact, the bulk of our experimental effort has gone into developing the methods (including methods of training and data handling) and correctly interpreting the results. An appropriate small animal model of ***visualizable***, ***sporadic, blood-borne micro-metastases of mammary carcinoma in the brain***, suitable for studies of immunotherapy, is essential for the field and was not previously available.

Below, we discuss each goal in turn. For each goal, our annual progress, as reported previously, is summarized and the direction of the new work is indicated (section **I**). Next, our work in the final period, not previously reported, is described in more detail (section **II**). The ***importance*** of the findings is explained, and appropriate ***follow-up*** studies are suggested.

TUMOR MODEL (tasks 1-3)

I. Summary of work previously reported in annual progress reports.

a. We adapted a method for injecting tumor into the carotid artery, chosen because this favors delivery of blood-borne tumor to the brain.

We first injected inert polystyrene beads, to confirm that the intracarotid injections did deliver tumor-sized objects in the expected pattern.

New work. Although the method was successful, it was very difficult for most lab members to learn. The steps we took to simplify the method, and to facilitate both training and data handling, are described in section **II.A.**, below.

b. As our model tumor, we used the well-characterized, highly metastatic *MATBIII* variant of the widely-used rat mammary carcinoma cell line, *13762*. We chose to use rat cells rather than human because, to study immunotherapy, we wanted to have a syngeneic system (where the tumor cell line was derived from the same species and strain as the host).

The cell line chosen was derived from the Fischer rat strain, the same strain we had used in many previous studies of immune regulation in the brain. Although the MATBIII cells were known to be metastatic in general, *brain* metastases had not been the focus of previous work with these cells.

c. To aid detection of micro-metastases in the brain, we caused the cells to constitutively express the lacZ reporter gene (*MATB/lacZ*), using methods we had previously used for other kinds of brain tumor cell lines (glioma, etc.; Lampson 92, 93). A simple histochemical stain for the lacZ gene product, E. coli-derived b-galactosidase (b-gal) allows detection of even single tumor cells in tissue sections, and facilitates quantitative analysis (Lampson 92, 93).

d. We have also introduced visualizable markers into other relevant cell lines:

- We transduced the original MATB cell line with an alternative marker, alkaline phosphatase (*MATB/ap*). Like the b-gal marker, this marker can also be detected by histochemistry. Having two cell lines with different markers will help with planned follow-up studies, as described below.

- We had obtained a different variant of the original 13762 cell line, passaged multiple times between blood and brain, as a gift from the late Dr. Peter Steck; we then transduced the cells with the lacZ marker (*M9/lacZ*).

- Separately, we had also transduced a rat B cell variant, YB2/0, with the lacZ marker (YB/lacZ).

The latter two cell lines provide a complementary migration pattern to the MATB cells, as described below.

e. We adapted our earlier methods as needed, to eliminate background staining in other organs (besides brain), where the tumor might metastasize. To correctly identify tumor in lung or lymph nodes, we needed to avoid background staining by the abundant phagocytes in those tissues.

Our tumor marker is *E. coli-derived* b-galactosidase (b-gal), which we detect by a simple and commonly-used histochemical assay on tissue sections. The main source of background in the assay is *mammalian* b-gal, a normal lysosomal enzyme, which is detected most readily in phagocytes. The key to specific detection is that the optimal pH differs for the two enzymes, with higher pH (pH 8) favoring detection of the marker enzyme. A second factor is timing: at pH 8, marker enzyme activity comes up sooner.

In practice, we showed that attention to the pH and timing of the reaction would allow us to avoid false positives in phagocytes of lung and lymph node, as confirmed by including appropriate negative control tissue in the assays.

New work. We continue to monitor the specificity of the histochemical reaction under all new conditions (simplified injection methods, different times of sacrifice, etc.). As controls for the stress of surgery, rats are given intracarotid injection of buffer instead of tumor cells. As positive controls for inflammatory or immune activity in the brain (including entry of inflammatory cells and activation of resident astrocytes and microglia), we continue to use brains from rats with autoimmune inflammation (*experimental allergic encephalitis, EAE*).

In parallel, we made practical observations about the staining behavior of the tumor cell lines as they grow in culture, which will be useful to others (as described below.)

f. In most of our previous work with the MATB/lacZ cell line (as detailed in the previous annual reports), we allowed as much time as possible for the tumor to grow, before sacrificing the rats. Our procedure, as approved by our Standing Committee on Animals, was to monitor the rats closely, and sacrifice them at the first signs of illness (ruffled fur and a hunched posture).

Using this as the endpoint, we defined the pattern of tumor growth as we varied the number of injected cells. We confirmed that, as would be expected, the tumor growth pattern was dose-dependent: As the cell dose was increased, the rats showed the first signs of illness sooner, and more tumor was seen in the brain.

New work. In our most recent work, we have emphasized earlier sacrifice times, for the reasons given in **g.**, below.

g. Our research focus is on the smallest tumor foci (*micro-tumor*) and strategies for delivery of therapy to micro-tumor sites. Our key aim has been to develop an appropriate model of sporadically distributed, *blood-borne* micro-metastases. An attractive feature of our model is that injected material enters vessels at *all* potential sites for tumor entry: vessels in the brain proper (parenchyma), meninges, and choroid plexus.

As we have reported, MATB/lacZ tumor that has entered vessels of the choroid plexus grows vigorously. When rats are allowed to survive until they show signs of illness, the ventricle can be filled with tumor and greatly increased in size (fig. 1).

Evaluation of the original slides suggested that tumor could invade the brain from the ventricle (fig. 1), a pathway that is also established for human tumor. This raised a question about the source of micro-tumor seen in the brain proper: Might it have entered from the ventricle, rather than from the blood?

New work. In our previous work, we took care to avoid the area next to the ventricle, when we were evaluating micro-tumor in the brain. Here, we addressed the question more directly, sacrificing rats before the tumor mass in the ventricle had begun to form.

We have now confirmed the presence of parenchymal micro-metastases where there is no tumor mass in the ventricle, and the ventricle is normal in size. This work is described in section **II. B.**, below.

In sum, in our most recent work, we continued our ongoing effort to simplify our methods, to make our model most useful to the field (**II. A.**, below). We then went on to confirm our interpretation that tumor could enter from the blood (**II. B.**, below).

II. Progress since the last report (work not previously reported)

A. Technical matters.

1. Surgery. Our surgical methods had been chosen to favor delivery of blood-borne tumor to the brain, and minimize the distribution to other organs. The methods were developed by a senior post-doctoral fellow, Dr. Yoichi Kondo. Dr. Kondo's previous work had been in rat brain ischemia; he brought to the project a detailed knowledge of the vasculature and excellent surgical skills. The method he developed involved ligating 3 different vessels, and was difficult for less experienced lab members to perform.

Convinced that the simplest possible method would be of greatest value to the field, we began to ask if all of the details of the full method were necessary.

a. In evaluating the original method, we had injected inert polystyrene beads before going on test tumor cells. However, analysis of the beads still required preparing the brain, cutting sections, etc. As part of the simplification, we wanted to have something we could follow right at the time of surgery.

As we asked questions about the injection methods, we found it useful to simply inject dye, rather than cells or beads, in the first rats. Injection of methyl green (3 %) allowed us to immediately see the course of injected material under varied surgical conditions; it was not necessary to first sacrifice the animal and remove the brain.

When methyl green is injected into the carotid artery by our original procedure, the dye can be seen to move along the artery. The eye and ear on one side turn dark green, as well as the nose and mouth area.

The pattern of injected dye was used as the first test of simplification. In different rats, the procedure was simplified, step by step, until only the left common carotid artery was isolated, and no side vessels were ligated. At each step, the eye and ear on one side still turned bright green. This suggested that, although the original method may optimize delivery to the brain, the quantitative gain might not be enough to justify the additional technical difficulty.

We then injected 10^5 inert polystyrene beads into the left carotid artery (of new rats), using the simplified procedure. In each of 2 rats, the yield of beads in the brain was at least as great as in 3 rats in which the same number of beads had been injected by the original method (fig. 2).

In comparing the old and new methods, the distribution of beads was similar. With both methods, beads were most concentrated on the side of tumor injection. (After beads were injected into the left carotid artery, most beads were seen on the left side of the brain.) Beads were seen in the vessels of the brain proper, meninges, and choroid plexus. The distribution among the different vessels was consistent with the number of vessels of each type. The distribution among different anatomical regions of the brain was consistent with their relative areas. These findings are consistent with the known blood flow pattern in the rat.

Conclusions.

- Injection of dye proved to be a good indicator of the final, quantitative results.
- We were able to greatly simplify the injection method, without sacrificing the yield of inert beads. The advantages are that the surgery is shorter and less stressful, both for

the rats and for the surgeon, rats are now only rarely lost during surgery, and training is much easier.

Cara Tripp, a recent college graduate and current technician, took the lead in testing the dye and simplifying the method; ***Dennis Meredith***, a Harvard medical student doing a year of research in the lab, injected and analyzed the beads.

b. Our experience in training may be useful to others. A common problem was that the trainee would stay in the rat room, trying rat after rat, becoming increasingly fatigued and frustrated. It proved very helpful to say, "just try one rat, no matter what happens; then come back and we'll talk it over, away from the rat room." It also was helpful to insist that, although it was understood that the procedure might not succeed, it should fail for a different reason each time, not the same reason as before.

With this policy, we began to solve problems one by one, trainees stopped repeating mistakes, the training interval was shortened, and many fewer rats were used.

c. One of the most difficult steps in the surgery is to insert the needle into the artery without piercing the artery. We wanted to develop a way to practice this step that did not waste rats. At first, we constructed a mock artery out of a piece of a latex glove (which had the desired stretchy elastic property). Dennis Meredith, the participating medical student, took this farther. He obtained some stretchy, elastic tubing and hooked it up to a pump, to simulate the blood flow. He has also constructed a small platform, to help hold the hand in position during the injection.

Importance. Simplifying the methods for learning and performing the intra-carotid injections is of value to our own lab, and will be to others in the field.

2. In vitro assay. In our model, detection of brain metastases depends on a histochemical stain for the lacZ reporter gene product, b-gal, expressed by our tumor cell line. It is important to monitor the cells' lacZ expression as they grow in culture. (In the course of long-term culture, it is always possible that any given gene product will be lost; if this were to occur, our first response would be to re-clone the cell line.)

In the course of routine monitoring, we noted that the degree of b-gal expression is reduced in sparse cultures. If the cultures are assayed when they are too sparse in the well, the full extent of b-gal expression is not revealed. The same result was seen with several different subclones of the original cell line.

Interpretation. With the method we use to transduce our cells with the lacZ gene (retrovirus), the gene is inserted randomly into the genome. Depending on the point of insertion, gene expression could certainly be influenced by "normal" metabolic processes of the growing cell. A related factor is that, as cells grow in culture, they "condition their medium," laying down extra-cellular matrix, secreting growth factors, etc. Either or both of these factors could cause differences in the level of b-gal expression at different culture density.

Importance. We have found that the b-gal marker is an excellent in vivo tumor marker and surrogate antigen (Lampson 92, 93). In practical terms, our observations about in vitro monitoring will be of use to ourselves and others.

B. Re-evaluating the tumor pattern. Using the simplified surgical methods described in **A.**, above, we went on to re-evaluate the pattern of micro-metastases in the brain proper, after intracarotid injection of tumor cells. Our goal was to confirm that tumor can enter the brain from the blood; it need not enter from a tumor-filled, distended ventricle. The key difference from our previous work, besides the simplified surgical methods, is that we sacrificed the rats much earlier.

Methods. In a series of studies, female Fischer rats received intracarotid injections of 1 x 10^6 MATB/lacZ cells, according to the simplified methods in **A.**, above.

Rats were sacrificed on days 0 through 7 by intracardiac injection of fixative (2 % paraformaldehyde), according to our usual methods (Lampson 92, 93).

Again following our usual methods: brains were removed, cryoprotected in sucrose and snap frozen, and 6u cryostat sections were cut through the brain. Sections at regular intervals were stained histochemically to reveal the b-gal+ tumor cells.

Stained sections were examined by light microscopy, and the location and appearance of all b-gal+ tumor cells were recorded.

Data handling. Originally, we made small drawings of tumor location as we looked at the slides, then recorded the data (tumor location, size, morphology, relationship to vessel, etc.) in tables. However, this became increasingly cumbersome as we added new rats and new properties of interest.

Our microscope is equipped for photomicrography and image analysis (Metamorph). As we analyze each slide, we have found it most helpful to mark the location of individual mets on a low power view, then take higher power views of the individual tumor foci (an example is seen in fig. 8). The slides are then organized into a Powerpoint presentation for each brain.

The medical student Dennis Meredith, the technician Cara Tripp, and two student-technicians, Maegan Hurley, and Sahar Zelkha, worked on the project as a team.

Results.

1. Tumor pattern in rats sacrificed on days 0 - 2.

Background information. In our model, we perfuse the rats with fixative, in order to optimize the morphology and the b-gal staining. A concern was that, when we perfused the rats, newly adherent cells might be washed out of the vessels.

In our previously-reported work, we addressed this concern by omitting the perfusion. In that work, each rat received an intracarotid injection of 10^7 MATB/lacZ cells, and was sacrificed 0-20 hours later. We found that, as expected, the tissue preservation and b-gal activity were compromised.

As an alternative way to detect tumor, we stained the sections with monoclonal antibody to keratin. (As we had confirmed earlier, for MATB/lacZ tumor growing in the brain, keratin can serve as an alternative tumor marker.) Keratin staining was stronger than b-gal staining, but both stains showed the same pattern: Tumor appeared as single cells and lines along the length of the vessels, similar to what had been seen with the polystyrene beads.

Here, we took a complementary approach. Although we feared that perfusion might wash the tumor out of the vessels, it was possible that (some) tumor would be lodged securely enough to avoid this.

Set-up. A total of 7 rats received the original MATB/lacZ cell line, and were sacrificed after intervals of 6 hours to 2 days. Serendipitously, an 8th rat received a subclone of the original line (subclone 210E), which we later noted had a greater tendency to form clumps. This rat was sacrificed at day 1.

Findings.

a. No tumor was detected in any of the 7 brains that received the original MATB/lacZ cell line, including 1 rat sacrificed at 6 hours, 1 sacrificed after 1 day, and 5 sacrificed after 2 days. Thus, our concern that early perfusion would wash out tumor may have been justified.

b. The rat receiving the 210E subclone (and sacrificed on day 1), did show b-gal+ tumor in the brain parenchyma. The greater tendency of these cells to clump may have contributed to the positive outcome. The clumps may have been more likely to be trapped; or the cells may have been more likely to adhere to the vessels, as well as to each other.

c. In the positive rat, we did *not* detect masses of tumor in the ventricles, or at other sites. Thus, this rat was suited to our goal of evaluating the tumor pattern in the absence of a tumor mass in the ventricle.

d. In the positive rat, b-gal$^+$ tumor was detected in several sites in the brain parenchyma (fig. 3).

- All of the tumor was seen on the side of the injection. (The tumor had been injected into the left common carotid artery, and all of the tumor was on the left side of the brain.)
- Within the left side, the tumor was widely and sporadically distributed. Examples are seen in fig. 3.
- The tumor appeared as individual cells or pairs. No masses were seen.
- In many cases, the tumor cell(s) appeared to be associated with a vessel. Examples are seen in fig. 4.

Interpretation. The results in this single rat were consistent with the observed distribution of keratin+ tumor in non-perfused rats (reported previously), the inert polystyrene beads, and the known blood-flow pattern of the rat: Within 1 day of injection, tumor was distributed widely within the hemisphere of injection; it was associated with vessels, and distributed in a pattern consistent with the blood flow.

2. Tumor pattern in rats sacrificed on days 3-7. A total of 8 rats received intracarotid injection of the original MATB/lacZ cell line, and were sacrificed from 3 - 7 days later. Analysis was as described above.

Findings.

a. In all, 4/8 (50%) of the rats sacrificed between days 3 and 7 showed b-gal+ tumor cells in the brain parenchyma. The % of positive rats was consistent within this interval (1/2 rats sacrificed on day 3, 1/1 on day 4, 1/3 on day 5 and 1/2 on day 7).

b. In each of the rats, we again did not see large masses of tumor in the ventricles (or at other sites); the ventricles were symmetrical and were not distended. Thus, the positive rats were suited to our goal of evaluating the tumor pattern in the absence of a tumor-filled, distended ventricle.

c. In the 4 tumor-bearing rats, all of the tumor was on the injection side. This is consistent with the known blood flow pattern, the behavior of blood-borne inert beads, the distribution of keratin+ tumor in non-perfused rats, and the distribution of tumor in a single perfused rat at day 1, all as discussed above.

d. The appearance of the tumor was more varied than what had been seen in rats sacrificed before day 3 (above). Now, in additional to single cells, some of which appeared to be vessel-associated, multi-cellular clusters were seen; these had not been detected earlier.

e. In some cases, the multi-cellular clusters of tumor cells, sometimes with together with other cells, could be seen inside vessels (fig. 5). In other cases, the relationship to a vessel (if any) was less clear (fig. 6).

Follow-up. Double-labeling for tumor and endothelial cells, using our previous methods (Lampson 94), will help to further define the relationship between tumor and vessels.

Interpretation. Metastatic breast cancer in the brain can appear as emboli trapped in the vessels (Takamura). Many of the multi-cell clusters had this appearance. The follow-up studies will suggest whether other clusters were also inside vessels.

f. Where several tumor cells were seen together, cell division or accumulation of additional cells from the blood may each have contributed.

Follow-up and interpretation. Use of MATB clones with two different markers (lacZ and alkaline phosphatase, respectively, which we have already prepared), can help distinguish between cell division and accumulation.

For example: The two cell lines can be injected together. If some clusters have (mostly) cells with the b-gal marker and other clusters have (mostly) cells with the alkaline phosphatase marker, this would imply that cell division, starting from a single tumor cell, had made a major contribution to the growth of each cluster.

3. Re-evaluation of rats sacrificed at later times. We re-examined a set of 6 brains from our previous work. These rats had received 1-3 x 10^6 tumor cells by the original surgical method, and had been sacrificed when they showed the first signs of illness, 9-11 days later.

These rats did have tumor-filled, enlarged ventricles on the injection side, as illustrated in fig. 8. In addition, tumor with these appearances was seen:

a. Clusters of cells, often appearing to be gathered around vessels, were seen (fig. 7). The cells appeared to cluster around the vessel, and not invade the adjacent brain.

Interpretation. Brain metastases from most non-lymphoid tumors, including breast cancer, have a characteristic growth pattern in the brain proper. If the tumor does enter the brain, it remains confined to the perivascular space, and does not enter the neuropil. The appearance of the vessel-associated clusters, with the cells accumulating around the vessel and not moving into the surrounding brain, is consistent with this expectation.

b. Having defined the early appearance of blood-borne tumor (fig. 4), we asked how many similar examples would be found in a later brain, when we looked for them specifically. We selected one brain with a large mass of ventricular tumor and searched all slides for evidence of vessels with only one or a few associated cells. In fact, we found many such examples, as illustrated in fig. 8.

Interpretation. The presence of the tumor-associated vessels shown in fig. 8 suggests that new tumor may continue to enter from the blood, several days after intra-carotid tumor injection.

In fact, we do not expect the original injected tumor to survive in the blood for this long; we would expect it to be trapped or killed. However, we do know that the tumor can metastasize to other organs (and have confirmed tumor in lymph nodes and lung following intracarotid injection). One explanation is that new tumor is being released into the blood from those sites. If this does occur, it would be an attractive feature of the model, parallel to what occurs in human patients.

c. Tumor was seen in meningeal vessels, in both the day 3-7 rats (which do not have massive tumor in the ventricle) and in the later rats. Larger masses in the meninges are seen with time. Again, this could reflect cell division or accumulation.

Interpretation. Although we have focused on tumor in the brain proper, meningeal metastases are also an important source of metastatic tumor for human breast cancer patients.

Overall interpretation. The work above was done to test our original interpretation of our tumor model: Did we, in fact, have blood-borne metastases? Taking all of our findings together (old and new rats):

- We confirmed the presence of tumor in cerebral vessels at day 1.
- We confirmed the presence of tumor in the brain proper, often associated with parenchymal vessels, at days 3-7 (before large tumors masses in the ventricle had formed).
- We found evidence of parenchymal vessels with freshly-entering tumor even at later times.

Other key findings were:

- Larger tumor masses in the brain proper appeared to cling to the walls of vessels; the tumor did not invade the brain. This is the expected behavior for most kinds of brain metastases.
- It appears that our model tumor can enter the brain by all three of the routes that have been identified for blood-borne cells. This idea is developed in the section below.
- The model is reproducible. The new work, including surgery and data handling, was done by an entirely new set of trainees.

Three routes for blood-borne cells. A recent review describes three routes by which blood-borne cells may enter the brain (Ransohoff): Through meningeal vessels, through micro-vessels in the brain proper, and -- something usually not emphasized -- through choroid vessels, moving to the CSF and then to the brain parenchyma. Our tumor model appears to display each of these routes:

- Inert beads are seen in vessels at each site (meningeal, parenchymal, choroid).
- Many small vessels with adjacent tumor cells are seen in the brain proper, both before and after large ventricular masses have formed.
- Tumor is seen in meningeal vessels, again both before and after ventricular masses have formed.
- In rats with tumor-filled ventricles, tumor cells do appear to invade the brain at the edge of the ventricle (fig. 1). It has been suggested that this route may be more common for metastatic tumor than was previously thought. Thus, although large ventricular tumor masses are not usually seen in human patients, the fact that our model tumor uses this route is relevant.

Moreover, it is likely that the cells can enter via choroid vessels even without a large tumor mass. Tumor cells (and beads) are seen in choroid vessels early, and lymphocytes use this route without forming a large mass in the ventricle (Ransohoff). In our model, we can study this at early times (before the ventricle mass has formed.)

In addition, our preliminary analysis of two other cell lines (one related to the MATB/lacZ line, and one a B cell line) provide examples of tumor that enters from the blood and does not form a mass in the ventricle, even at later times.

4. Variation from rat to rat. Although brain metastases are increasingly common among breast cancer patients, they are not detected in all patients, either clinically or even at autopsy. The factors that determine which patients will develop brain metastases are not known.

Given the heterogeneity of human patients, variability in the incidence and other features of brain metastases is not surprising. This same heterogeneity contributes to the difficulty of identifying the most important variables.

In this context, it is of interest that we also see variability in our rats: Under a given set of experimental conditions (cell dose, time of sacrifice), only half of the rats sacrificed at day 3-7 showed parenchymal brain metastases. It may be that evaluation of more slides would reveal parenchymal metastases in additional rats. Indeed, more sensitive methods might also reveal them in more human patients. However, the point remains that there is at least a quantitative differences among the rats, as there is among human patients.

We are working with highly inbred rats in a closely-monitored specific pathogen-free (spf) facility. Intriguingly, we have seen a parallel variability in another experimental model in use in the lab, the common model of autoimmunity, experimental autoimmune encephalitis (EAE). Rats given the same disease-inducing injection vary greatly in their clinical scores.

In seeking explanations for this variation, we have selected three possibilities that would be relevant to human patients:

- Variations in the estrus cycle from rat to rat. This can be tested simply by monitoring the cycle, and by manipulating estrogen levels pharmacologically. (This project was suggested by the medical student, Dennis Meredith, who will now pursue it under the PIs guidance.)

- Variations in surgical stress. This can be tested by monitoring the time for each surgery, especially the time during which the artery is being manipulated, and by varying the times intentionally.
- Variations in the levels of pro-inflammatory cytokines (perhaps as a result of one of the factors above). Pro-inflammatory cytokines, such as tumor necrosis factor (TNF), can increase entry of blood-borne cells to many tissues, including brain (Lampson 94). Our third approach will be to ask if CNS delivery of TNF increases the yield of blood-borne metastases in our model.

Importance.

- The variables we will test would be relevant to human patients.
- Any pre-clinical test of therapy will be more easily interpreted if a consistent number of metastases is present from rat to rat.

TUMOR THERAPY (tasks 4-6)

I. Summary of previous work.

Our proposed approach to therapy focused on enhancing a T cell-mediated immune response to micro-metastases in the brain. As described in our annual progress reports, we adapted, evaluated, and compared different methods to activate T cells: ***a)*** Immunization of the tumor-bearing rat to stimulate endogenous T cells. ***b)*** Immunization of separate rats, harvest of their T cells, growth of the T cells in vitro, and finally adoptive transfer of fluorescently-labeled T cells into the tumor-bearing rats. Technical adaptations, as well as advantages and disadvantages of each method, for experimental work and for human patients, were included in the annual progress reports. (These same methods will be of use in the studies described below.)

In parallel, work in our own laboratory and also work in the field has greatly influenced our thinking about T cells in particular, and immunotherapy in general. In her published work, the PI has consistently stressed the variety of immune and inflammatory attack mechanisms, and the importance of "matching the therapy to the tumor type and site." Our own most recent experience, plus that of others, has directed our attention to two attack mechanisms that complement the potential activity of T cells, but have received less attention in the context of brain-metastasizing breast cancer: **A**. Attack by phagocytes (monocytes, macrophages, microglia), especially at the earliest stages of metastatic tumor entry to the brain. **B**. A novel combination of cell-mediated and antibody-mediated approaches. Details are given below.

II. Progress in the last year (work not previously reported)

A. Activated phagocytes.

Methods. This work began as a follow-up analysis of tumor-bearing brains from rats sacrificed 1-7 days after injection of tumor into the carotid artery (described above). As part of the initial analysis of these brains, we had stained slides at regular intervals through the brains to reveal micro-metastases.

Now we took slides adjacent to those that had been stained to reveal tumor. The new slides were stained with monoclonal antibody OX6, according to our published methods.

In the brain, this antibody stains activated microglia and other phagocytes. Few such cells are seen in the normal brain. However, activated microglia are seen in many pathological and experimental settings. Indeed, their presence is a general and sensitive indicator of abnormality in the brain, as the PI and many others have shown (Lampson 03b).

Results, interpretation, and follow-up. In the brain proper of tumor-injected rats, we saw scattered foci of a few darkly stained OX6+ phagocytes. The distribution was consistent with the suggestion that these cells might be associated with micro-metastases, or might be marking places where metastatic tumor had entered, but not survived.

Our previous findings with intracerebrally-injected (rather than blood-borne) tumor supports this idea. In those studies, where we were able to identify tumor and OX6+ activated phagocytes in the same slides, we confirmed that the activated phagocytes were seen adjacent to tumor, and not in tumor-free areas (Dutta 03).

Here, we will follow up in a similar way, using double-staining to better define the relationship between micro-metastases and activated phagocytes.

Implications for immunotherapy. If activated phagocytes are indeed responsible for the initial destruction of newly-arrived blood-borne brain mets, this suggests an immuno-therapeutic approach that may deserve more attention.

In fact, the role of activated phagocytes in the brain is controversial. In different experimental conditions, phagocytes can indeed attack tumor. However, under other conditions, they can secrete growth factors and other molecules that promote cell growth (reviewed in Lampson 03b). Our focus on, and ability to examine, these issues at the earliest stages of tumor entry from the blood will add to this field.

B. A novel approach. In the PI's view, tumor immunity and autoimmunity are two sides of the same coin. The wealth of information about T cell-mediated *auto*immunity can give insight that is relevant to T cell-mediated tumor immunotherapy (Lampson 04). Thus, we complement our work in tumor immunotherapy with work in a common model of T cell-mediated autoimmunity, experimental allergic encephalitis (EAE). In fact, our own recent work in the EAE model (under separate funding), together with others' work, have greatly influenced our thinking about tumor therapy. Taken together, the most recent work makes us acutely aware of the difficulty of increasing T cell-mediated immune response in the brain. This in turn has encouraged us to think about alternative forms of immune attack. Below, some of the key findings are summarized in **1.**, and the way we have responded in our thinking is described in **2.**

1. Key points behind our current thinking.

a. In our own work, we found that manipulations that were expected to *increase* the immune response in EAE did not do so – and might even be *protective*. ***i)*** Injection of gamma-interferon (IFN-g), which is immune-activating in many contexts, did not exacerbate EAE. ***ii)*** Injection into the brain of substance P (SP), a neuropeptide that is immune-activating in many contexts, was *protective* in EAE (Zhang 03). In fact, these findings are consistent with many other reports of unexpected clinical outcomes in this model.

b. The difficulty of *increasing* T cell-mediated activity in the brain, at least in EAE, is also supported by other kinds of findings. Indeed, in the most common EAE model, immunization of the rats produces only one episode of autoimmune attack; subsequent immunizations do not produce further autoimmune activity.

The implication in a tumor context is sobering: Even if one initially "breaks" tolerance to tumor antigen with a tumor vaccine, the tolerance may be "spontaneously" re-established, and the patient refractory to further immunization.

c. Work in a brain tumor context also draws attention to the difficulty of *increasing* T cell-mediated immune activity in the brain. Many ways in which a tumor can evade T cell-mediated immune activity (for example, through the Fas / FasL pathway) have now been identified (reviewed in Lampson 03b).

d. Complementing this work in disease models, work in disease-free brains also points to the difficulty of increasing T cell-mediated immune activity in the brain. Provocatively, it has been proposed that the regulatory environment of the brain, like that of the eye, favors an antibody-mediated response, rather than a T cell-mediated response (Harling-Berg 99).

e. In her published work, the PI has consistently stressed the variety of immune and inflammatory attack mechanisms, and the importance of "matching the therapy to the tumor type and site."

To date, much work in brain tumor immunotherapy has focused on a T cell-mediated response, and that was indeed the focus of our original application. However, for the reasons given above, we have recently paid particular attention to alternative ways of attacking blood-borne brain mets (other than T cell-mediated attack). The role of activated phagocytes in attacking the earliest micro-mets is discussed above. A novel combination of cell-mediated and antibody-mediated approaches is discussed below.

2. A novel approach. Although recent focus has been on the T cell-mediated response, there has also been much work in antibody-mediated attack of tumor. A major difficulty for brain tumors is that normal antibody molecules are too large to cross the blood-brain barrier. The normal solution to this problem is to truncate the antibody. However, making the antibody molecule smaller also sacrifices some of the structural features that contribute to its biological activity against tumor.

An intellectual fruit of our work on this project has been a novel approach to the challenge of delivering whole antibody molecules to the brain. The basis is: Although antibody *molecules* are stopped by the blood-brain barrier, antibody forming *cells* (activated B cells) can enter the brain. Indeed, antibody forming cells are found in the brain in many common neurologic disorders (including autoimmune disease).

We are now working to direct antibody forming *cells* to the brain, and to ask if they can be exploited to deliver therapy (antibody or novel molecules) to blood-borne micro-metastases. The model we have developed here gives us the context to try our ideas. (This is the topic of the R21 application being submitted February 1.)

KEY RESEARCH ACCOMPLISHMENTS

- We have developed a novel, appropriate model for blood-borne metastatic mammary carcinoma in the brain. The model has several attractive features.
    - The smallest micro-metastases can be readily detected in tissue sections.
    - The micro-metastases have an appropriate sporadic distribution in the brain.
    - The tumor can enter the brain by the three appropriate vascular routes: vessels in the brain proper, meninges, or choroid plexus.
    - The syngeneic system is suitable for studies of immunotherapy.
    - The methods are sufficiently simple for general use. All new work reported was done by a new team of trainees.
- We draw attention to the variety of immune effector mechanisms, especially:
    - The potential of activated phagocytes to attack newly arriving blood-borne cells.
    - The potential of migratory, activated B cells to deliver antibody (or other agents) to micro-tumor sites in the brain.
- In a broader context, the PI has continued her efforts to encourage fresh thinking about brain tumor trials ("Strengthening the Bridge from Bench to Bedside").

REPORTABLE OUTCOMES not previously reported

Cell lines. Of the cell lines we have prepared, the ***MATB/lacZ*** cell line is the best characterized, and can be used by other investigators. The other cell lines mentioned can be made available when we have completed their initial characterization.

The PI has routinely made tumor cell lines and hybridomas available to the community through the American Type Culture Collection (ATCC). According to ATCC records, since 1998, 500 (five hundred) vials of her material have been distributed to other investigators.

New research Support.

Awarded: R21 NS45765, NINDS/NIH, 3/1/03 - 2/31/05, Role: P.I.

This project uses the new metastatic tumor model to study the interaction between tumor migration and lymphocyte migration in the brain.

Pending: A new R21 application (February 1 deadline) proposes to use the new model to test the hypothesis that B cells can deliver therapy to micro-metastases in the brain.

Special sessions.

1. "Strengthening the Bridge from Bench to Bedside," special 2-hour session at the annual meeting of the Society for Neuro-Oncology (SNO), proposed and discussion led by the PI, Montreal, 2004, 77 attendees.

Session summary distributed to all attendees, and to be posted on the Society website. (Session summary is enclosed.)

2. The PI conducted a similar 1-hour discussion for NINDS program directors, at NIH, January, 2004.

New abstract.

Meredith, Tripp, Kapoor, Kondo, and Lampson, "Visualizable, sporadic, blood-borne micro-metastases of mammary carcinoma in the rat brain." In preparation for Era of Hope Meeting, 2005.

A full length manuscript describing the model is in preparation.

Updated citations and special issues (previously reported).

Glick RP and Lampson LA, eds. Brain Tumor Immunotherapy. Special issue, *J. Neuro-Oncol.* 2003, 64: 1-191.

Lampson LA, editor. Animal models of CNS tumor. *J Neuro-Oncol* 2001, 53: 275-318. (A special section of the journal, with papers invited and edited by the PI.)

Lampson, LA. Basic Principles of CNS immunology. In Winn, HR, ed., *Youman's Neurological Surgery, 5th edition*, WB Saunders, 2003, pp. 673-688.

Lampson LA: Immune regulation in the brain: Lessons from autoimmunity, implications for brain tumor therapy. *Human Brain Tumors*. F. Ali-Osman ed, Humana 2004, pp 175 - 205.

CONCLUSIONS

Brain-metastasizing breast cancer is an important clinical problem. Our work in this project has contributed practically and conceptually.

Practically, we have provided a model for visualizable, blood-borne micro-metastases with an appropriate sporadic distribution in the brain. The syngeneic system is suitable for studies of immunotherapy (as well as many other therapies.) The methods are suitable for trainees to learn.

Conceptually, we have worked to broaden the approaches being taken.

- Although there is growing interest in brain tumor immunotherapy, the field has tended to focus on particular topics. In our work on this project, and in general, we have sought to draw attention to additional and complementary aspects of the immune response. For example:

- Most work to date has emphasized immunotherapy for primary brain tumors (glioma, etc.) rather than blood-borne metastases. Work on "mets" has typically involved direct injection of a melanoma cell line into the brain. Our focus on blood-borne brain metastases of mammary carcinoma adds to this field.

- To date, much work in brain tumor immunotherapy has emphasized the *initiation* phase of the immune response: identifying tumor antigens, stimulating an immune response with tumor vaccines. Our emphasis on the effector phase at the tumor site adds to this field.

- Much recent work in tumor immunotherapy has emphasized the T cell-mediated immune response. In parallel, other labs have emphasized manipulation of antibody molecules so that that they can cross the blood brain barrier. Our current goal of using antibody forming *cells* to deliver antibody to the brain is a novel approach.

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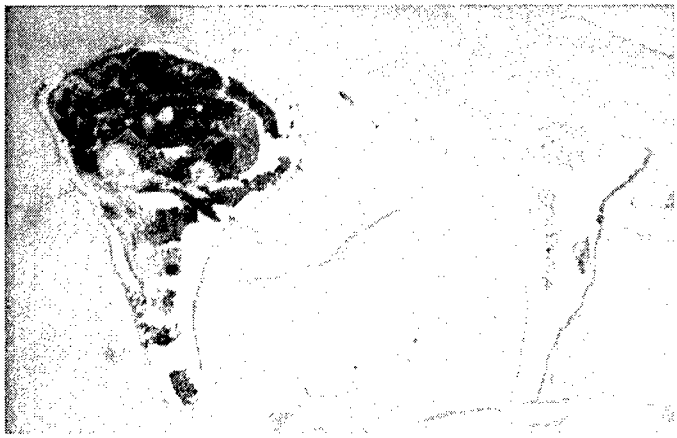
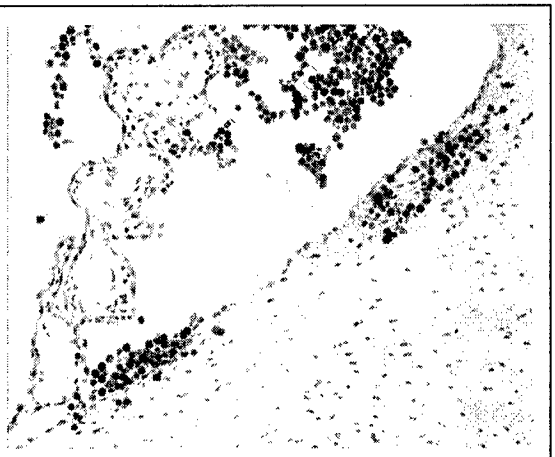
Zhang Y, Lampson LA. A model to study local immune control, especially the role of SP, in EAE. (Abstract) *American Association of Neuropathologists* (*AANP*) Annual Meeting, Orlando, 2003.

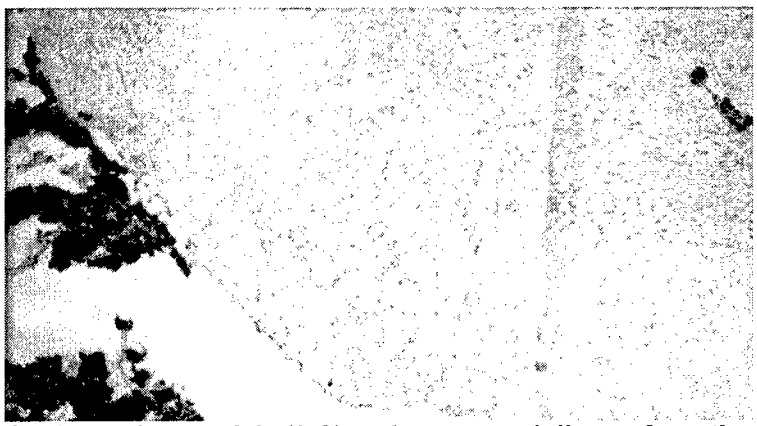
Fig. 1. Tumor at edge of ventricle.



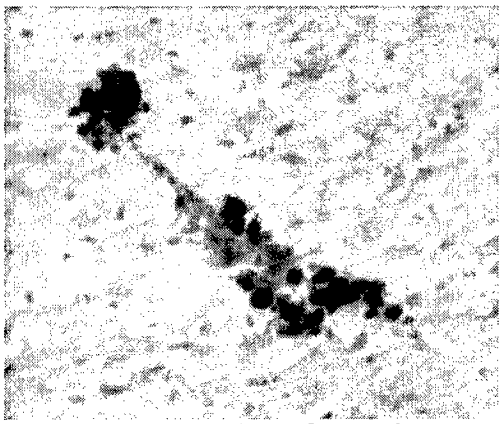
A. Distended, tumor-filled ventricle on injection side.



B. Tumor at edge of ventricle.



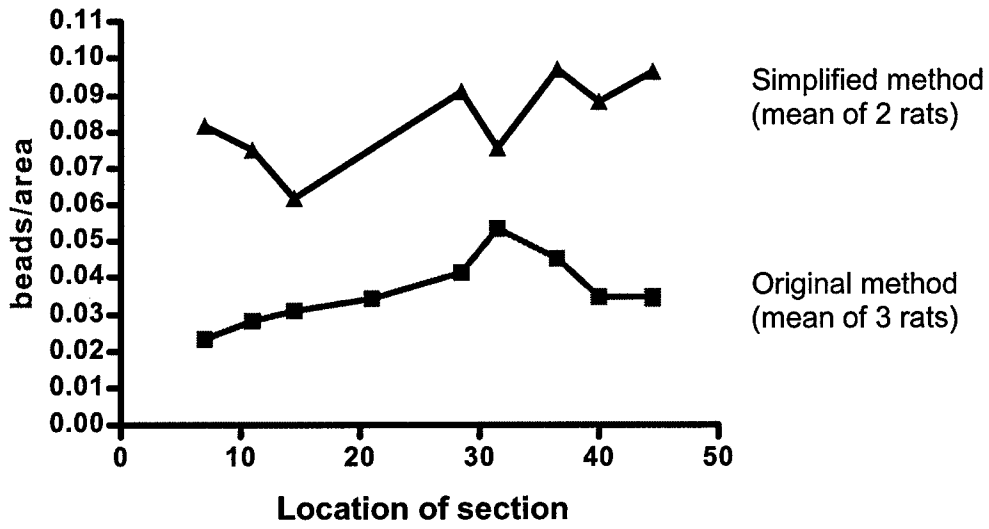
C. Tumor in ventricle (*left*) and at a vessel distant from the ventricle edge (*right*).



D. Higher power view of vessel-associated tumor shown in C.

Figure illustrates characteristic appearance of MATB/lacZ tumor in the brain at the time rats show first signs of illness after intra-carotid injection of tumor cells. (For doses of 0.5 - 3 x 10^6 tumor cells, rats first become ill after 9-12 days.) In each panel, tumor appears bright blue after histochemical stain for the b-gal tumor marker. (Tumor is black in these pictures.) **A. Ipsilateral lateral ventricle.** Tumor had been injected into the left carotid artery. At sacrifice, the left lateral ventricle is filled with tumor and distended. Some tumor is also seen in the opposite ventricle, but much less (not shown). **B. Edge of ventricle.** Scanning around the edge of the ventricle reveals locations where tumor appears to be entering the adjacent brain. **C. Vessel-associated tumor**. Tumor at a vessel distant from the ventricle. **D.** Higher power view of the vessel-associated tumor shown in **C**. A key goal of our new studies was to confirm, in rats that did not have a tumor mass in the ventricle, that micro-metastases could indeed enter the brain proper from the blood.

Figure 2. Beads per unit area after intracarotid injection.



Rats received intracarotid injection of 10^5 polystyrene beads, using either our original surgical method (top line) or the simplified method (bottom line). The location of each section was identified according to the corresponding plate number in the rat brain atlas (Paxinos and Wilson). The number of beads in each section was counted with the aid of an eyepiece grid, the area of the section was measured (Metamorph), and the density was computed. Note the presence of beads in all sections, and that the yield was at least as great with the simplified method.

Fig 3. Sporadic distribution of blood-borne tumor on day 1.

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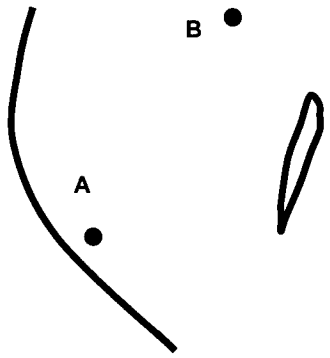
left edge, left ventricle
 
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<td>Figure shows location of b-gal$^+$ tumor (black dots) with respect to major landmarks (edge of section, ventricles, midline) in a series of slides. (Representative micrographs of tumor are seen in fig. 4.) Rat had been sacrificed 1 day after intracarotid injection of b-gal$^+$ tumor cells; sections through the brain were stained to reveal b-gal$^+$ tumor.</td>
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 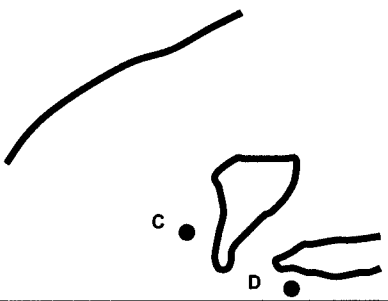
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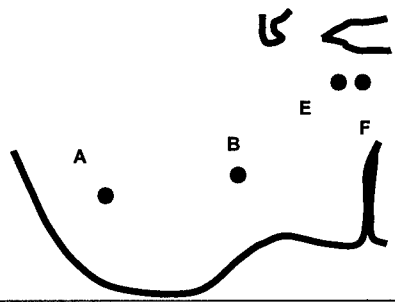
bottom, midline
 
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 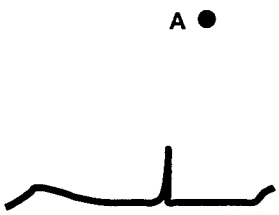
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 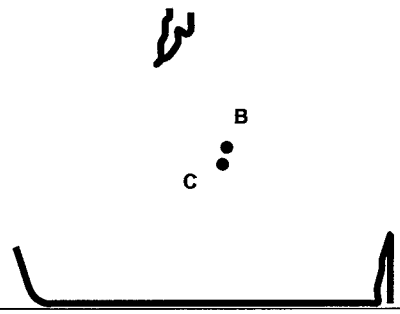
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 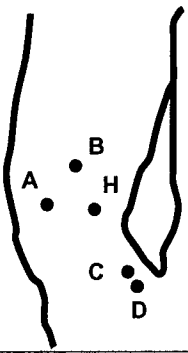
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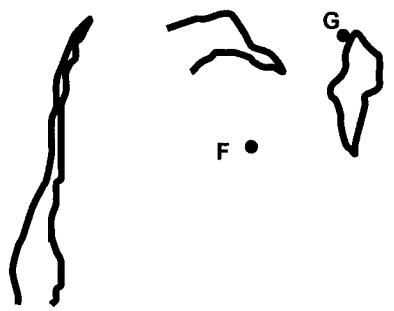
left ventricle
 
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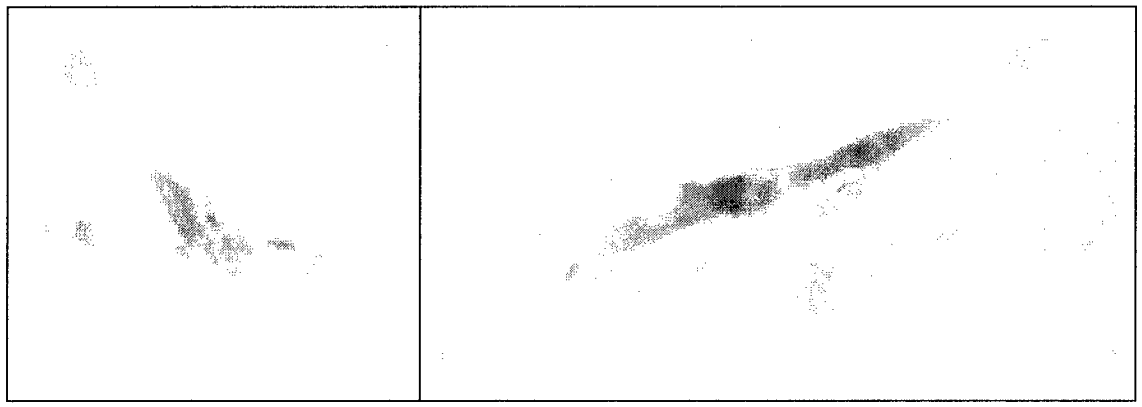
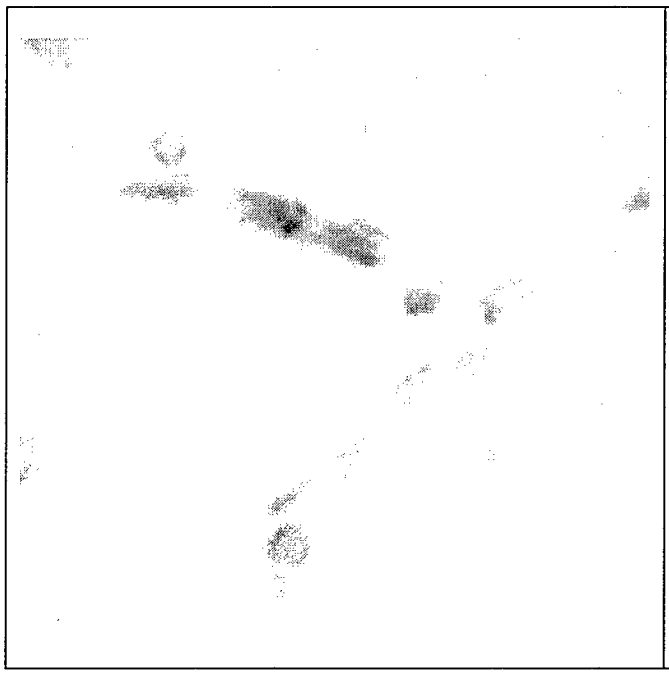
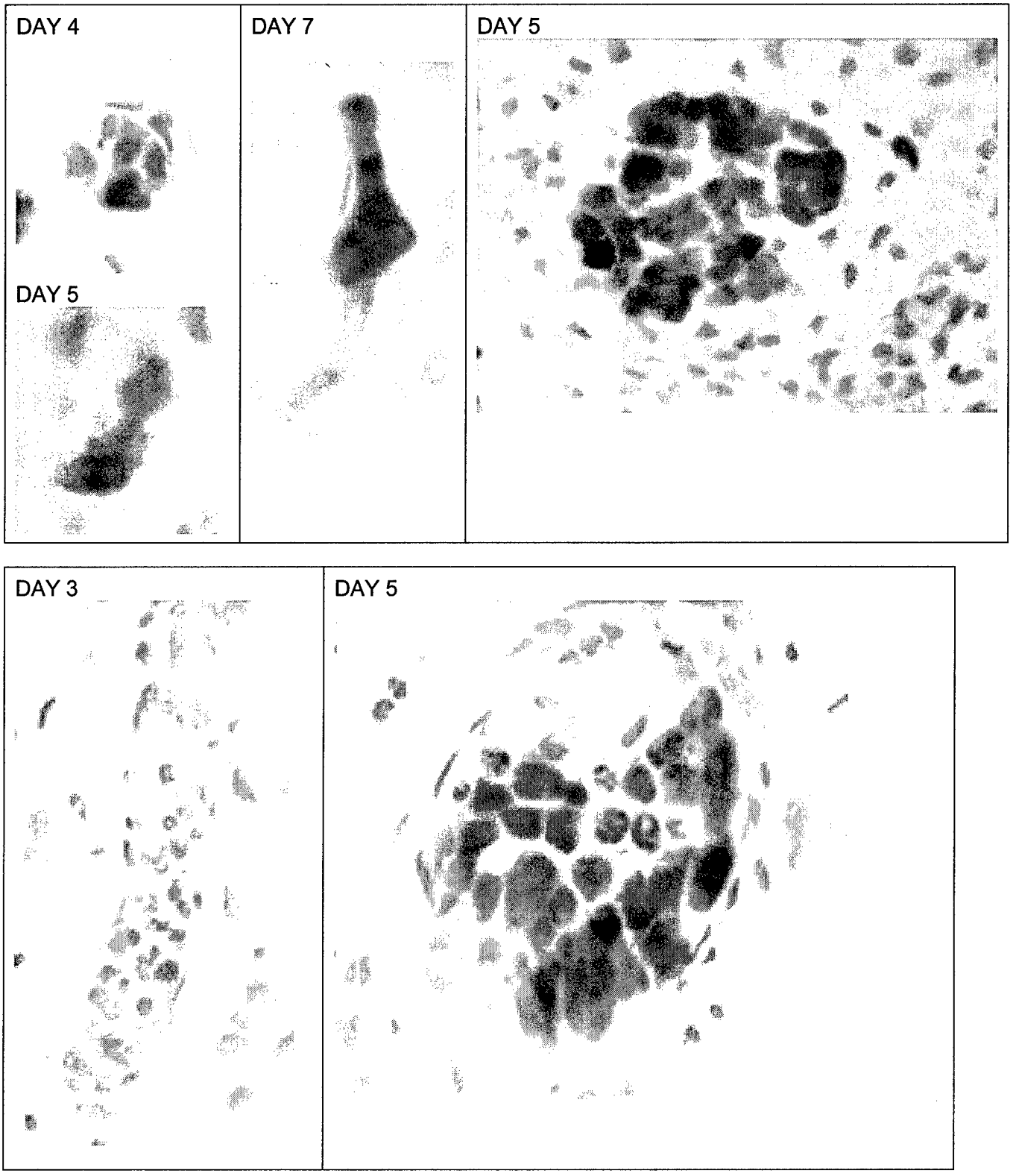
Fig. 4. Examples of vessel-associated tumor on day 1.

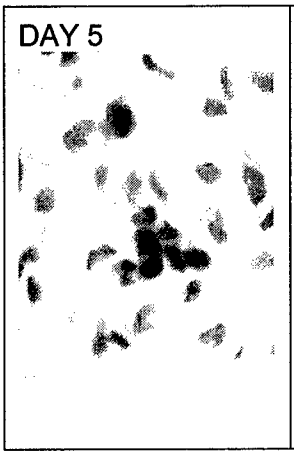




Micrographs are taken from the same tissue depicted in Fig. 3. Figure illustrates vessel-associated b-gal+ tumor cells, which appear bright blue after histochemical stain for b-gal.

Figure 5. Tumor emboli. Figure illustrates tumor-filled vessels from rats sacrificed 3-5 days after intracarotid injection of 1 x 10^6 MATB/lacZ tumor cells. Tumor appears bright blue after histochemical stain for b-gal. Panels are arranged according to size of the embolus.





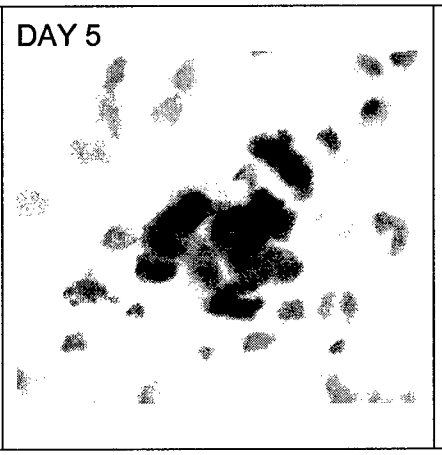


Fig. 6. Tumor clusters, days 5-7.

Figure illustrates tumor cell clusters in the brain parenchyma, where the relationship to the vessel is less apparent than in fig. 5. Tumor is bright blue after histochemical stain for b-gal; light hematoxylin counterstain. Examples are arranged according to size.